Involvement of cholesterol in the inhibitory effect of dimethyl-β-cyclodextrin on P-glycoprotein and MRP2 function in Caco-2 cells

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Abstract **We compared the inhibitory effect of various cyclodextrins (CyDs) on P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) function and examined the contribution of cholesterol to the inhibitory effect of 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) on the efflux activity of the function in Caco-2 cell monolayers. Of various CyDs, DM-β-CyD significantly impaired the efflux activity of P-gp and MRP2. DM-β-CyD released P-gp and MRP2 from the monolayers in the apical side's transport buffer and decreased the extent of cholesterol as well as P-gp and MRP2 in caveolae of Caco-2 cell monolayers, but not caveolin and flotillin-1. On the other hand, DM-β-CyD did not change *MDR1* and *MRP2* mRNA levels. Therefore, these results suggest that the inhibitory effect of DM-β-CyD on P-gp and MRP2 function, at least in part, could be attributed to the release of these transporters from the apical membranes into the medium as secondary effects through cholesterol-depletion in caveolae after treatment of Caco-2 cell monolayers with DM-β-CyD.**

Key words: Cyclodextrin; Cholesterol; P-glycoprotein; Multidrug resistance-associated protein 2; Caveolae; Caco-2

1. Introduction

Intestinal P-glycoprotein (P-gp) and multidrug resistance-associated protein 2 (MRP2) are ATP-dependent multidrug efflux pumps, which can serve as active secretion systems or absorption barriers by transporting drugs from the apical membrane of intestinal cells into the lumen [1,2]. The substrates transported by P-gp and MRP2 include a variety of structurally and pharmacologically unrelated compounds such as some anticancer agents, steroidal hormones, calcium channel blockers, immunosuppressive agents and β-blockers [3–6].

Cyclodextrins (CyDs) are host molecules which form inclusion complexes with lipophilic drugs as guests and thus have been utilized for improving their water solubility and dissolution rates [7]. Recently, several hydrophilic CyD derivatives have been used, e.g. methylated, 2-hydroxypropylated, sulfobutylether and branched CyD derivatives [8–11]. In the cell biology field, methyl-β-cyclodextrin (M-β-CyD) and 2-hydroxypropyl-β-CyD (HP-β-CyD), which are introduced the substitution groups randomly, have been widely used as cholesterol-depletion agents [12].

We recently demonstrated that 2,6-di-*O*-methyl-β-cyclodextrin (DM-β-CyD) significantly increased the low aqueous solubility and oral bioavailability of tacrolimus, an immunosuppressive agent, in rats and the improvement in oral bioavailability was due not only to its solubilizing effect but also, at least in part, to its inhibitory effect on the P-gp-mediated efflux of tacrolimus from intestinal epithelial cells [13]. P-gp is reported to be localized in caveolae, flask-shaped invaginations, in which cholesterol and glycosphingolipids are abundant in multidrug-resistant (MDR) cells and in brain capillaries [14,15]. In addition, caveolae containing caveolin-1 reside in the small intestinal brush border membranes [16]. Then, we postulated that DM-β-CyD may interact with cholesterol in plasma membranes of enterocytes, resulting in changes of caveolae function, which may eventually impair the efflux activity of P-gp and MRP2 in enterocytes if these transporters reside in caveolae. To clarify the hypothesis in the present study, therefore, we studied the inhibitory effects of hydrophilic CyD derivatives on P-gp and MRP2 efflux activity in Caco-2 cell monolayers and the effect of DM-β-CyD on the extent of P-gp, MRP2 and cholesterol in caveolae of apical membranes and their release from the monolayers into the apical side's transport buffer.

2. Materials and methods

2.1. CyDs

2-Hydroxypropyl-α-cyclodextrin (HP-α-CyD), HP-β-CyD and 2-hydroxypropyl-γ-cyclodextrin (HP-γ-CyD) with an average degree of substitution (DS) of the hydroxypropyl group of 4.0, 4.8 and 4.8, respectively, as well as DM-β-CyD (DS = 14) were obtained from Nihon Shokuhin Kako. Sulfobutylether β-CyDs (SBE4- and SBE7-β-CyDs, DS = 3.9 and 6.2, respectively) were supplied by CyDex. M-β-CyD (DS = 10.5–14.7) was purchased from Sigma Chemicals.

2.2. Cytotoxicity and transepithelial electrical resistance (TEER)

Caco-2 cells (passages 40 and 60) were obtained from the American Type Culture Collection and cultivated as reported previously [13].

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Abbreviations: AP-to-BL, apical to basolateral; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester; BL-to-AP, basolateral to apical; CyDs, cyclodextrins; DS, degree of substitution; DM-β-CyD, 2,6-di-*O*-methyl-β-cyclodextrin; HP-α-CyD, 2-hydroxypropyl-α-cyclodextrin; HP-β-CyD, 2-hydroxypropyl-β-cyclodextrin; HP-γ-CyD, 2-hydroxypropyl-γ-cyclodextrin; HPLC, high performance liquid chromatography; MRP2, multidrug resistance-associated protein 2; M-β-CyD, methyl-β-cyclodextrin; MBS, MES-buffered saline; MDR, multidrug-resistant; P_{app}, apparent permeability coefficient; P-gp, P-glycoprotein; RT-PCR, reverse transcription polymerase reaction; SDS, sodium dodecyl sulfate; TEER, transepithelial electric resistance

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The effects of CyDs on viability and integrity of Caco-2 cell monolayers were measured as reported previously [17]. In brief, intestinal mitochondrial enzymatic activity was assayed using a Cell Counting Kit (WST-1 method) from Wako Pure Chemical Industries. All TEER values of Caco-2 cell monolayers grown on Transwell® (6-well) were measured by using Millicel®-ERS Voltmeter (Millipore). The TEER value of Caco-2 cell monolayers was 761.4 ± 5.1 Ω/cm^2.

2.3. Transport studies

The effects of treating with CyDs on the transport of [^{3}H]mannitol (specific activity, 19.7 Ci/μmol, New England Nuclear) and the effects of pretreatment with CyDs on the rhodamine 123 (Molecular Probes) and BCECF (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; Molecular Probes) across Caco-2 cell monolayers were studied as described previously [13]. Briefly, [^{3}H]mannitol was added to the apical side's transport buffer in the absence and presence of CyDs and incubated for 60 min. On the other hand, the apical membranes were pretreated with 10 mM CyDs for 30 min for the transport studies of rhodamine 123 and BCECF. After washing the apical membranes, rhodamine 123 (5 μM) or 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethylester (BCECF-AM; 5 μM) was added to the apical or basolateral side's transport buffer. For other inhibitors, the efflux of rhodamine 123 or BCECF was determined in the presence of 5 μM cyclosporin A or 200 μM indomethacin in the apical and basolateral side's transport buffers without pretreatment. The radioactivity of [^{3}H]mannitol was counted in an Aloka LSC-3500 liquid scintillation counter. The concentrations of rhodamine 123 and BCECF in the transport buffer were determined by a high performance liquid chromatography (HPLC) and a Hitachi F-4500 fluorophotometer (excitation and fluorescence wavelengths were 439 nm and 525 nm, respectively), respectively. The HPLC conditions were the same as that reported previously [13]. The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = (dQ/dt)/(A \cdot C_0)$$

where dQ/dt is the flux across the monolayer (mol/s), A is the surface area of the membrane (cm^2), and C_0 is the initial drug concentration (mol/ml).

2.4. Release of membrane components

Caco-2 cells in 35 mm dishes were cultured in growth medium for 15 days before use. Prior to experiments, Caco-2 cell monolayers were incubated for 24 h in growth medium supplemented with 10% FCS containing [^{3}H]cholesterol (5 μCi/ml, Amersham-Pharmacia Biotech). The cell monolayers were washed three times with transport buffer and incubated in transport buffer containing CyDs for 30 min. The concentrations of [^{3}H]cholesterol and total proteins in the transport buffer were determined with an Aloka LSC-3500 liquid scintillation counter and bicinchoninic acid reagent from Pierce Chemical with bovine serum albumin as a standard, respectively. In some experiments, 10 mM DM-β-CyD-containing transport buffer preloaded with 1.6 mM cholesterol by incubation with free cholesterol for 7 days was used.

2.5. Western blotting

The expressions of P-gp, MRP2, caveolin and flotillin-1 were detected by Western blotting. In brief, the samples were separated by 7%, 10% and 15% sodium dodecyl sulfate (SDS)-PAGE for the assay of P-gp and MRP2, flotillin-1 and caveolin, respectively, and then transferred onto PVDF membranes (NEN Life Science Products). The membranes were blocked with 5% skim milk, and then probed with C219 anti-human P-gp (Signet Laboratories), M_2I-4 anti-MRP2 monoclonal antibody (Alexis Biochemicals), C13630 anti-caveolin polyclonal antibody (BD Transduction Laboratories) or Clone 18 anti-flotillin-1 mouse IgG_1 antibody (BD Transduction Laboratories). After a wash, the membranes were incubated with peroxidase-conjugated secondary antibody and then the specific protein bands were detected using the ECL Western blotting analysis kit (Amersham-Pharmacia Biotech). The bands were detected using the Lumino-image analyzer LAS-1000 plus (Fujifilm).

2.6. Semiquantitative reverse transcription polymerase reaction (RT-PCR)

The apical membranes of Caco-2 cell monolayers were treated with CyDs for 30 min. Then, the membranes were scraped off and the cells were lysed. Total RNA was extracted from the lysates using a RNeasy Mini Kit (Qiagen). Next, the samples were treated with deoxyribonuclease (1 U) and ribonuclease inhibitors (35 U) and complementary DNA was synthesized by reverse transcription using the specific reverse primers against human *MDR1*, *MRP2* or *β-actin* gene and reverse transcriptase (SuperScript II). Then, PCR was conducted using the specific forward primers against the human *MDR1*, *MRP2* and *β-actin* genes. PCR was performed for 25 cycles of denaturation at 94°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 120 s. The amplified products were analyzed on 2% low melting temperature agarose gels containing 0.1 μg/ml of ethidium bromide.

2.7. Caveolae isolation

Low-density caveolae-enriched domains were isolated by a carbonate-based fractionation method as described previously [18]. In brief, Caco-2 cell monolayers cultured in 150 mm dishes were scraped into 2 ml of 0.5 M sodium carbonate (pH 11) and homogenized extensively using a Potter-Elvehjem type homogenizer, Polytron tissue grinder and a sonicator. The resulting homogenate was brought to 45% sucrose by the addition of 2 ml of 90% sucrose in MES-buffered saline (MBS; 25 mM MES and 150 mM NaCl, pH 6.5) and overlaid with two layers of 35% and 5% sucrose in MBS containing 0.25 M carbonate (4 ml each). The gradient was then centrifuged at 200 000 × g for 18 h using a Beckman SW41Ti rotor. For the analysis of the resulting gradient, 12 fractions (each 1 ml) were collected from the top to the bottom of the gradient. 10 μl of each fraction were subjected to SDS–PAGE for the detection of P-gp, MRP2 and caveolin, respectively. On the other hand, the cholesterol mass of each fraction obtained from the sucrose gradient was determined using a Cholesterol Kit Wako® (Wako Pure Chemical Industries).

3. Results

3.1. Cytotoxicity and monolayer's integrity

Fig. 1 shows the effects of CyDs on viability of Caco-2 cells. Tween 20, a non-ionic surfactant, was employed as a positive

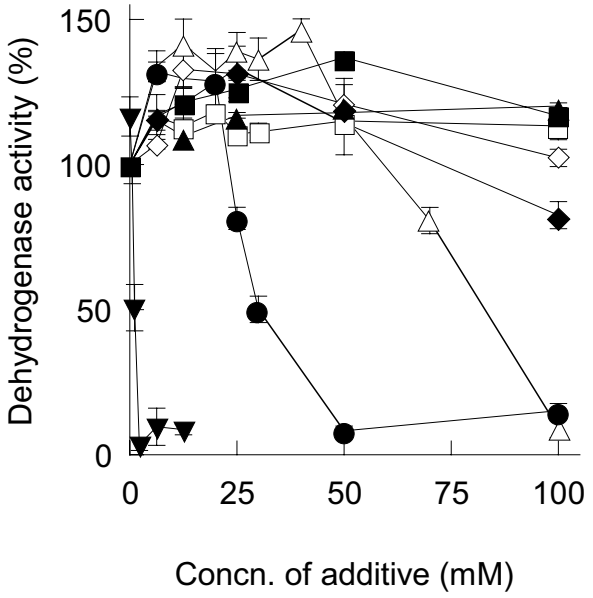


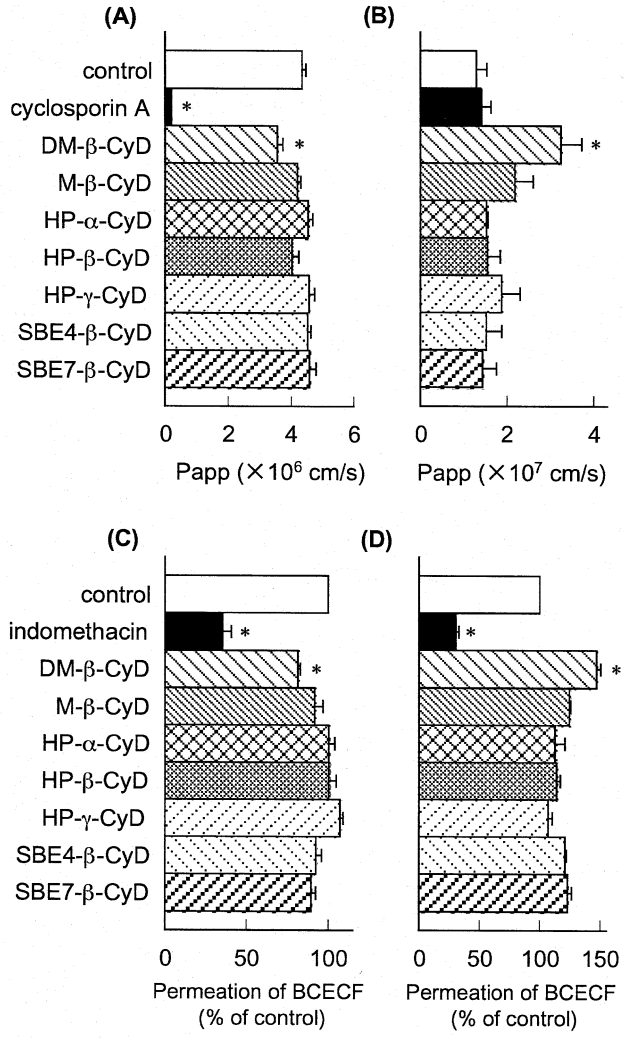
Fig. 1. Cytotoxicity of CyDs and Tween 20 in Caco-2 cells in Hanks' balanced salt solution (HBSS; pH 7.4) at 37°C. (Closed circles), DM-β-CyD; (open triangles), M-β-CyD; (closed triangles), HP-α-CyD; (open squares), HP-β-CyD; (closed squares), HP-γ-CyD; (open diamonds), SBE4-β-CyD; (closed diamonds), SBE7-β-CyD; (closed inverted triangles), Tween 20. Cells were washed three times with HBSS (pH 7.4) and then incubated for 60 min with 100 μl of HBSS containing CyDs and Tween 20 at various concentrations at 37°C. After three washes with HBSS to remove CyDs and Tween 20 again, cell viability was assayed by the WST-1 method. Each point represents the mean ± S.E.M. of six experiments.

Table 1
Effects of CyDs treatment on TEER and permeation of [^{3}H]mannitol (50 nM) through Caco-2 cell monolayers

System	Caco-2	
	TEER % of initial value	P_{app}[a] $\times 10^7$ (cm/s)
control	116.7 ± 2.3	2.22 ± 1.7
with DM-β-CyD (10 mM)	107.6 ± 1.5	2.33 ± 0.4
with DM-β-CyD (15 mM)	103.2 ± 1.5	2.35 ± 0.2
with M-β-CyD (15 mM)	104.5 ± 0.2	1.63 ± 0.3
with M-β-CyD (50 mM)	83.3 ± 1.4*	2.60 ± 0.4
with HP-α-CyD (100 mM)	120.0 ± 1.2	2.02 ± 0.6
with HP-β-CyD (100 mM)	126.4 ± 0.3	1.81 ± 0.0
with HP-γ-CyD	146.4 ± 1.5*	1.43 ± 0.2
with SBE4-β-CyD (100 mM)	113.8 ± 1.8	2.87 ± 0.3
with SBE7-β-CyD (100 mM)	92.4 ± 1.3*	2.31 ± 0.6

*$P < 0.05$ versus control.
[a] P_{app} of AP-to-BL [^{3}H]mannitol permeation.

control and eventually provided almost complete cytotoxicity even at 8.9 mM. HP-α-CyD, HP-β-CyD, HP-γ-CyD, SBE4-β-CyD and SBE7-β-CyD were not cytotoxic to Caco-2 cells up to 100 mM. On the other hand, DM-β-CyD and M-β-CyD markedly reduced the viability of these cells above 50 mM and 100 mM, respectively. Next, we evaluated the integrity of tight junctions of the monolayers as a molecular barrier, and the effects of CyDs on the TEER and the ability of mannitol to permeate the monolayers were studied. As shown in Table 1, 100 mM HP-α-, HP-β-, HP-γ-, SBE4-β- and SBE7-β-CyDs as well as 10 mM and 15 mM DM-β-CyD and 15 mM M-β-CyD did not lower the TEER value, while 50 mM M-β-CyD did. In addition, both DM-β-CyD and M-β-CyD changed the P_{app} values of [^{3}H]mannitol, a paracellular transport marker, only very slightly at a concentration of 15 mM, but 50 mM M-β-CyD significantly increased the values (Table 1). On the other hand, 100 mM HP-α-, HP-β- and HP-γ-CyDs and sulfobutylether β-cyclodextrins did not markedly increase the values (Table 1). Taken together, these results suggest that M-β-CyD and DM-β-CyD, particularly DM-β-CyD, strongly interact with Caco-2 cell monolayers, resulting in a decline in the monolayer's integrity at higher concentrations.



3.2. DM-β-CyD, but not M-β-CyD, inhibits P-gp and MRP2 function

To elucidate whether CyDs inhibit the P-gp- and MRP2-mediated efflux of substrates from Caco-2 cell monolayers, the effects of various CyDs on basolateral to apical (BL-to-AP) and apical to basolateral (AP-to-BL) transports of rhodamine 123 and BCECF, representative P-gp and MRP2 substrates, respectively, were examined. The results were compared with those for cyclosporin A and indomethacin, typical P-gp and MRP inhibitors, respectively. Here the monolayers were pretreated with 10 mM CyDs for 30 min to avoid direct interaction between the substrates and CyDs as well as the cellular damage, whereas they were treated with the inhibitors in the presence of the substrates. In addition, it is also noteworthy that CyDs provided neither cytotoxicity nor non-specific paracellular transport at concentrations up to 10 mM during 30 min of incubation (Fig. 1 and Table 1). As shown in Fig. 2A,B, it is apparent that cyclosporin A markedly decreased

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Fig. 2. Inhibitory effects of various agents on BL-to-AP (A,C) and AP-to-BL (B,D) permeation of rhodamine 123 (A,B) and BCECF (C,D) through Caco-2 cell monolayers. The apical membranes of Caco-2 cell monolayers were pretreated with CyDs at 10 mM for 30 min at 37°C. After a wash of the apical membranes, rhodamine 123 (5 μM) or BCECF-AM (5 μM) was added to the basolateral or apical side's transport buffer. For other inhibitors, the effluxes of rhodamine 123 and BCECF were determined in the presence of 5 μM cyclosporin A and 200 μM indomethacin in the apical and basolateral sides' transport buffers without pretreatment. The concentrations of rhodamine 123 and BCECF were determined by a HPLC and a fluorescence spectrophotometer. Each value represents the mean ± S.E.M. of three experiments. *$P < 0.05$ versus rhodamine 123 or BCECF alone.

the BL-to-AP permeation in the monolayers, but increased the AP-to-BL permeation of rhodamine 123 only very slightly, although the reason for no change in AP-to-BL permeation remains unknown. Of CyDs, DM-β-CyD inhibited the BL-to-AP transport by one-fifth and augmented the AP-to-BL transport of rhodamine 123 approximately 2.5-fold, compared to the control. The pretreatments with other CyDs had much less effect on the permeation of rhodamine 123.

Fig. 2C,D show the effects of CyDs on MRP2 function in Caco-2 cell monolayers. Here BCECF-AM, a lipophilic derivative of BCECF was used. In the monolayers, BL-to-AP efflux and AP-to-BL influx were inhibited and promoted by pretreatment with DM-β-CyD, respectively, although the inhibitory effect of DM-β-CyD on the BL-to-AP efflux was markedly lower than that of indomethacin. Other CyDs changed the efflux and influx of BCECF only very slightly. On the

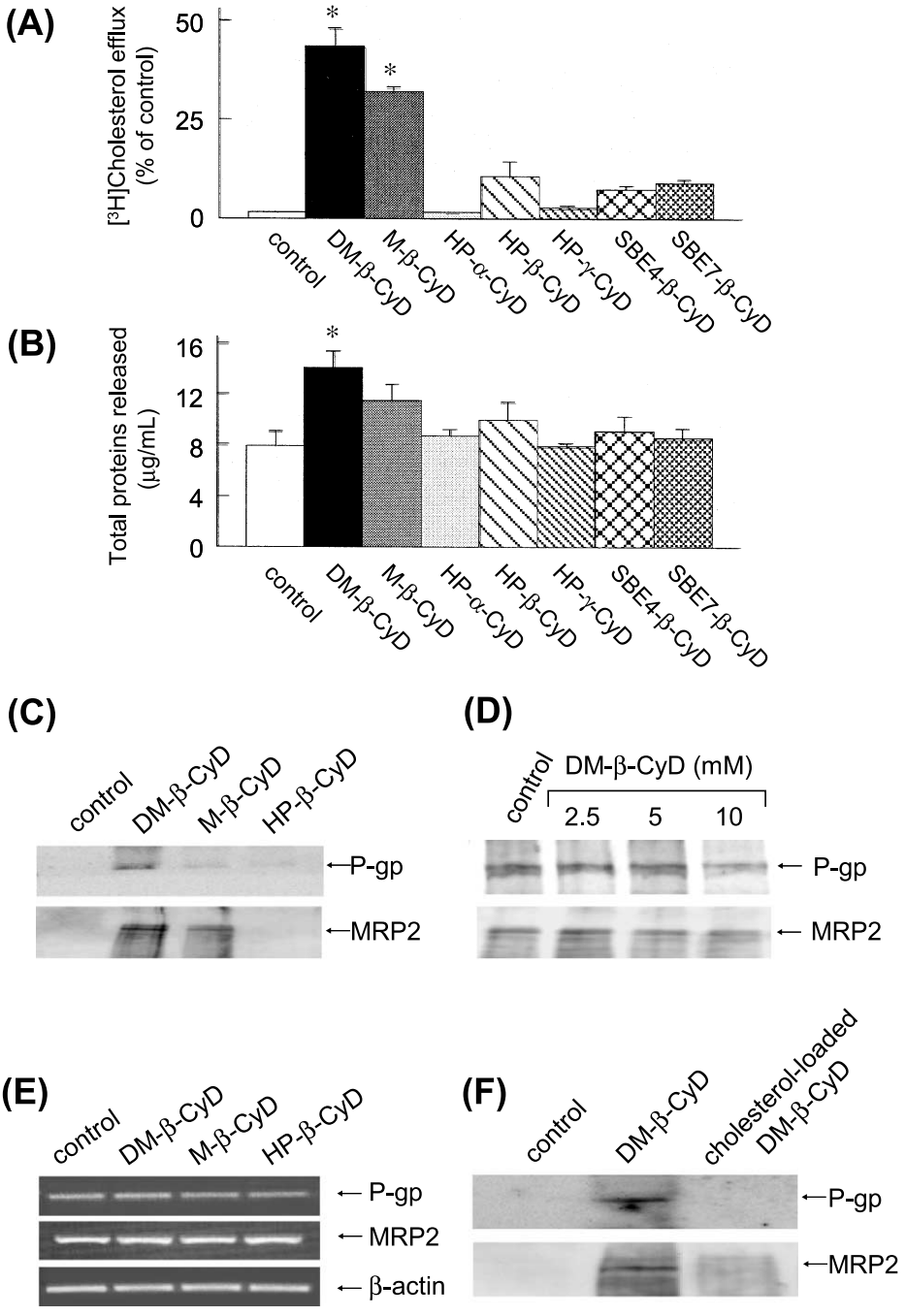

Fig. 3. Effects of CyDs on the release of [3H]cholesterol (A) and total proteins (B) from Caco-2 cell monolayers. 100% represents total amounts of [3H]cholesterol labeled in Caco-2 cell monolayers. Each value represents the mean ± S.E.M. of 3–11 experiments. $*P < 0.05$ versus control (without CyDs). C: Immunoblotting of P-gp and MRP2 released in the apical side's transport buffer of Caco-2 cell monolayers. D: Immunoblotting of P-gp and MRP2 in Caco-2 cell monolayers. The monolayers were treated with 10 mM CyDs for 30 min at 37°C and then total proteins and P-gp, MRP2 levels in the apical side's transport buffer and Caco-2 cell monolayers were assayed by the bicinchoninic acid reagent and immunoblotting, respectively. E: *MDR1* and *MRP2* mRNA levels in Caco-2 cell monolayers. The monolayers were treated with 10 mM CyDs for 30 min at 37°C and then *MDR1* and *MRP2* mRNA levels in the cells were assayed by RT-PCR. F: Effects of an addition of cholesterol to DM-β-CyD-containing apical side's transport buffer on the release of P-gp and MRP2 in the buffer of Caco-2 cell monolayers.

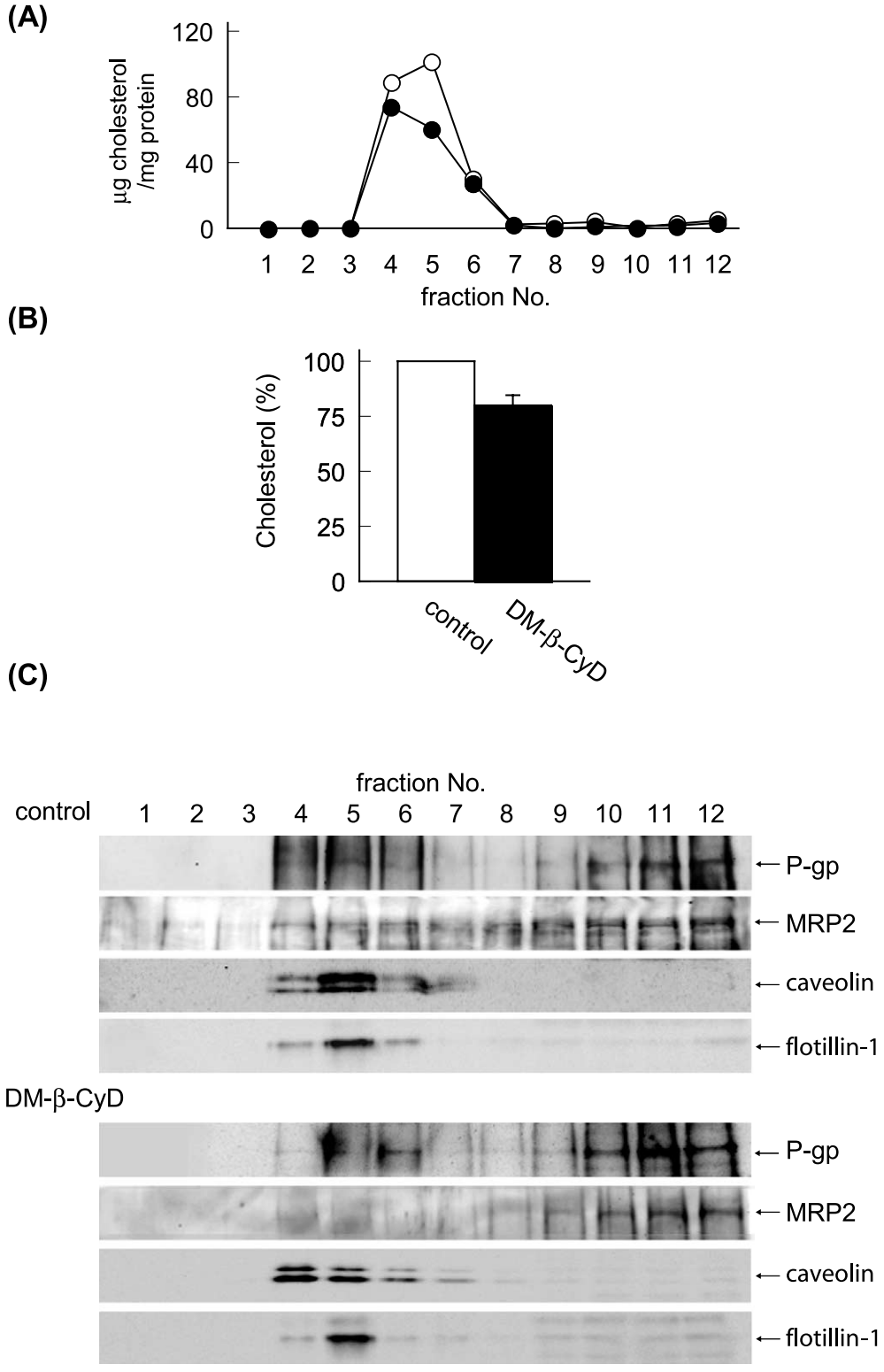

Fig. 4. A: Localization of cholesterol in caveolae of Caco-2 cell monolayers. The amount of cholesterol in each fraction after sucrose gradient centrifugation was determined with a Cholesterol Kit Wako®. (Open circles), control; (closed circles), DM-β-CyD. B: Effect of DM-β-CyD on the extent of cholesterol in caveolae fraction of Caco-2 cell monolayers. Bars refer to percent permeation as compared with control which was arbitrarily defined as 100%. Fractions 4–6 were defined as caveolae fraction. Each value represents the mean ± S.E.M. of three experiments. C: Effect of DM-β-CyD on P-gp and MRP2 localization in caveolae and non-caveolae fractions in Caco-2 cell monolayers. The monolayers were treated with 10 mM DM-β-CyD for 30 min at 37°C, caveolae were isolated, and the P-gp, MRP2, caveolin and flotillin-1 expressions were assayed by immunoblotting.

other hand, the impairment of AP-to-BL transport of BCECF following the treatment with indomethacin might be attributed to the inhibitory effect on MRP3, which resides in basolateral membranes of Caco-2 cell monolayers. These results indicate that of all the CyDs used here DM-β-CyD significantly inhibit the P-gp- and MRP2-mediated efflux from Caco-2 cell monolayers.

3.3. DM-β-CyD releases cholesterol as well as P-gp and MRP2 from Caco-2 cell monolayers

It is well recognized that β-CyDs release biological membrane components such as cholesterol and phospholipids from erythrocytes and nasal epithelium cells, resulting in release of proteins from cell membranes [19,20]. First we examined whether CyDs release cholesterol from the apical membranes of the monolayers. The treatment with DM-β-CyD and M-β-CyD raised the cholesterol level released from the monolayers (Fig. 3A), consistent with the results reported by Ohtani et al. who examined the release of lipids from erythrocytes [19]. Indeed DM-β-CyD increased the release of [^{3}H]cholesterol approximately 30-fold and 1.4-fold, compared with negative control and M-β-CyD, respectively (Fig. 3A). These results imply that DM-β-CyD attenuates P-gp and MRP2 function through cholesterol depletion, at least in part, in the apical membranes.

To gain an insight into the mechanism for the inhibitory effect of DM-β-CyD on P-gp and MRP2 function, we examined whether P-gp and MRP2 are released from the monolayers into the apical side's transport buffer. At first, we compared the treatment effects of CyDs (10 mM) for 30 min on the release of total proteins from the apical membranes of Caco-2 cell monolayers. As shown in Fig. 3B, the greatest effect on the release of total proteins was shown by DM-β-CyD, i.e. DM-β-CyD, M-β-CyD and HP-β-CyD released total proteins approximately 1.8, 1.5 and 1.3-fold higher than the negative control, respectively. It is important to note that the bands corresponding to P-gp and MRP2 were clearly observed in the apical side's transport buffer after treatment with 10 mM DM-β-CyD, compared to that with M-β-CyD and HP-β-CyD (Fig. 3C), but the bands corresponding to caveolin and flotillin-1, marker proteins of caveolae, were not observed (data not shown), indicating that the presence of the transporters in the buffer is not due to the contamination of cells removed from culture dishes. In addition, DM-β-CyD reduced the P-gp and MRP2 levels in Caco-2 cell monolayers (Fig. 3D) in a concentration-dependent manner, although the effect of DM-β-CyD on P-gp has been already reported [13]. The results of RT-PCR analysis demonstrated that the treatment of apical membranes of Caco-2 cell monolayers with DM-β-CyD, however, did not change the band densities corresponding to *MDR1* and *MRP2* mRNA (Fig. 3E), indicating that DM-β-CyD affects a transcription of P-gp and MRP2 only very slightly. Besides, an addition of free cholesterol to DM-β-CyD-containing buffer completely inhibited the release of P-gp and MRP2 into the apical side's transport buffer (Fig. 3F), suggesting that the ability of inclusion complexation of DM-β-CyD with cholesterol is involved in the release of the transporters. These results suggest that the transport activity of P-gp and MRP2 depends on the amount of cholesterol in the apical membranes and/or the release of P-gp and MRP2 induced a lowering in the cholesterol content from the apical membranes of Caco-2 cell monolayers.

3.4. Inhibitory effect of DM-β-CyD on P-gp and MRP2 function refers to caveolae

Caveolae are reported to enrich cholesterol and glycosphingolipids [21] and to exist on the plasma membrane of Caco-2 cells and enterocytes [16,22]. We examined the effects of DM-β-CyD on the localization of cholesterol in Caco-2 cell monolayers. Cholesterol was observed in fractions 4–6, consistent with the localization of caveolin (Fig. 4A), suggesting that caveolae reside in Caco-2 cell monolayers under the present experimental conditions. In addition, DM-β-CyD lowered the cholesterol level in caveolae (Fig. 4A,B). P-gp and MRP2 existed in both low and high density fractions, suggesting that these proteins resided in both caveolae and non-caveolae fractions of Caco-2 cell monolayers (Fig. 4C). The treatment with 10 mM DM-β-CyD for 30 min slightly decreased P-gp and MRP2 levels in caveolae, but not in non-caveolae (Fig. 4C). In addition, we confirmed that the treatment with DM-β-CyD scarcely affected the caveolin and flotillin-1 levels in caveolae of Caco-2 cell monolayers. Taken together, these results suggest that DM-β-CyD is likely to impair P-gp and MRP2 function through a release of P-gp and MRP2 from the apical membranes of Caco-2 cell monolayers into the transport buffer.

4. Discussion

We previously reported that DM-β-CyD inhibited P-gp function in Caco-2 cell monolayers [13]. The present study demonstrates that DM-β-CyD impaired not only P-gp but also MRP2 function in Caco-2 cell monolayers, probably through the release of these transporters from the monolayers, resulting from the extraction of cholesterol from the caveolae of the monolayers.

It is noteworthy that DM-β-CyD, and not other CyDs, significantly impaired the efflux activity of P-gp and MRP2 in the monolayers (Fig. 2). On the other hand, M-β-CyD and HP-β-CyD, which are widely used as cholesterol-depletion agents, attenuated the efflux activity only very slightly. The distinction in the inhibitory effect among these CyDs could be attributed to a difference in inclusion ability, which was greater for DM-β-CyD than M-β-CyD and HP-β-CyD, e.g. the solubilizing effect of β-CyDs on cholesterol indeed increased in the order of HP-β-CyD $<$ M-β-CyD (DS = 10.5–14.7) $<$ DM-β-CyD (DS = 14) (data not shown), probably resulting from the increase in hydrophobic space of the CyD cavity [23]. Importantly, these results strongly suggest that a slight difference in the DS value in methylated β-CyD may significantly affects the cellular function with a different magnitude.

The inhibitory effect of DM-β-CyD on the efflux activity of P-gp and MRP2 may result from the release of the transporters from the apical membrane of the monolayers into the apical side's transport buffer via cholesterol depletion in caveolae. This hypothesis may be supported by the results that β-CyDs changed their message levels only very slightly (Fig. 3E) and DM-β-CyD decreased the levels of P-gp, MRP2 and cholesterol in caveolae of the monolayers (Fig. 4C). The result that there was no change in a transcription of *MDR1* and *MRP2* may be due to impermeability of β-CyDs through plasma membranes. The impermeability of β-CyDs can be adequately explained by the release of P-gp, MRP2 and cholesterol from the monolayers into the apical side's buffer.

It is well known that CyDs interact with lipids stronger than proteins [24]. As expected, the activity of DM-β-CyD to release cholesterol from the Caco-2 cell monolayers was strongest, followed by M-β-CyD (Fig. 3A). This order was concomitant with that of the inhibitory effect on the transporter's function (Fig. 2). In addition, there were good inverse correlations between cholesterol efflux and BL-to-AP transport of rhodamine 123 and BCECF (data not shown) and an addition of cholesterol to DM-β-CyD-containing buffer provided neither the release of P-gp and MRP2 (Fig. 3F) nor the impairment of P-gp and MRP2 function (data not shown) in Caco-2 cell monolayers. Thereby, we presumed that the inhibitory effect of DM-β-CyD on P-gp and MRP2 function relates to caveolae. As a result, we could confirm that almost all of the cholesterol existed in caveolae and the cholesterol level in caveolae was decreased by the treatment with DM-β-CyD (Fig. 4A,B). Likewise, the extent of P-gp and MRP2 in caveolae was decreased by the treatment with DM-β-CyD (Fig. 4C), although that of caveolin and flotillin-1 was not changed by that with DM-β-CyD. Therefore, we propose that the ability of DM-β-CyD to release P-gp and MRP2 from the monolayers can be ascribed to the secondary effect on the basis of the release of cholesterol from caveolae of the apical membranes, which may lead to the inhibitory effect of DM-β-CyD on P-gp and MRP2 function in Caco-2 cell monolayers. However, this selective effect of DM-β-CyD to release proteins is tempting to speculate the possibility that DM-β-CyD may release P-gp and MRP2 from non-caveolae domains of Caco-2 cell monolayers and concomitantly may allow the transporters to move from caveolar to non-caveolae domains to replenish them. Elaborate studies are further required to reveal the mechanism for the effect of DM-β-CyD to release proteins from apical membranes of Caco-2 cell monolayers.

Does cholesterol retaining in the apical membranes of Caco-2 cell monolayers directly regulate P-gp and MRP2 function? To address the question, we examined the effect of fillipin, caveolae-dispersing agent, on P-gp and MRP2 function in Caco-2 cell monolayers. As a result, the treatment of the monolayers with 5 μg/ml of filipin failed to inhibit BL-to-AP transport of rhodamine 123 and BCECF in Caco-2 cell monolayers (data not shown), suggesting that cholesterol does not regulate P-gp and MRP2 function in a direct manner [25]. However, cholesterol is reported to impart stability to perturbation of bilayer organization by P-gp and moreover this led to altered protein function in reconstitution system [26]. In addition, other possibilities in terms of the inhibitory effect of DM-β-CyD on P-gp and MRP2 function cannot be ruled out: the change in membrane fluidization [27–29] and P-gp ATPase activity [30]. Further study regarding the detailed mechanism for the inhibitory effect of DM-β-CyD on the transporter's function should be performed.

In conclusion, of various CyDs, DM-β-CyD markedly impaired P-gp and MRP2 function and the inhibitory effect of DM-β-CyD on the function, at least in part, could be attributed to the release of these transporters from the apical membranes into the medium in terms of cholesterol-depletion in caveolae of Caco-2 cell monolayers.

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